Role of Analytical Ultracentrifugation in Assessing the Aggregation of Protein Biopharmaceuticals

Steven A. Berkowitz[1]

[1]Department of Analytical Development, Biogen Idec Inc, 14 Cambridge Center, Cambridge, MA 02142

ABSTRACT

In developing and manufacturing protein biopharmaceuticals, aggregation is a parameter that needs careful monitoring to ensure the quality and consistency of the final biopharmaceutical drug product. The analytical method of choice used to perform this task is size-exclusion chromatography (SEC). However, it is becoming more and more apparent that considerable care is required in assessing the accuracy of SEC data. One old analytical tool that is now reappearing to help in this assessment is analytical ultracentrifugation (AUC). Developments in AUC hardware and, more importantly, recent developments in AUC data analysis computer programs have converged to provide this old biophysical tool with the ability to extract very high resolution size information about the molecules in a given sample from a simple sedimentation velocity experiment. In addition, AUC allows sample testing to be conducted in the exact or nearly exact liquid formulation or reconstituted liquid formulation of the biopharmaceutical in the vial, with minimal surface area contact with extraneous materials. As a result, AUC analysis can provide detailed information on the aggregation of a biopharmaceutical, while avoiding many of the major problems that can plague SEC, thus allowing AUC to be used as an orthogonal method to verify SEC aggregation information and the associating properties of biopharmaceuticals.

KEYWORDS: Protein aggregation, analytical ultracentrifugation, size-exclusion chromatography, SEDFIT

INTRODUCTION

In the development of biopharmaceuticals, which are predominately protein-based compounds, the problem of aggregation looms as a key point of concern to scientists involved in cell culture, purification, and formulation. Hence, having appropriate analytical tools that are capable of detecting and quantifying biopharmaceutical aggregates is important. Although several analytical techniques are available to potentially provide this information, size-exclusion chromatography (SEC) is the method of choice. Its ease of use, the simplicity of its physical separation mechanism, its ability to measure low levels of aggregation with very small amounts of material, and the high speed in which separations occur, using relatively simple hardware, yield an inexpensive technique with high sample throughput. In most cases, SEC works well, achieving good size separations and valid aggregation information with high precision. Nevertheless, despite these positive attributes, care still must be exercised in assessing the validity of SEC data. When inaccuracies occur it is frequently because of the nonspecific interactions of the biopharmaceutical and its aggregates with the chromatographic material in the high performance liquid chromatography (HPLC) column or the HPLC-column hardware, especially the column frits.[1-5] To confirm the accuracy of SEC data, analytical ultracentrifugation[6-10] (AUC) can now be used. In addition, AUC can serve as an important characterization tool for studying the biophysical solution properties of a biopharmaceutical and its aggregates when SEC analysis is not feasible or when a direct assessment of the state of aggregation of a biopharmaceutical in the initial sampled vial is desired. This latter point is important because the SEC mobile phase frequently does not correspond to the initial liquid formulation of the biopharmaceutical being analyzed. In addition, during SEC, the initial concentration of the injected biopharmaceutical is greatly reduced. Such changes in the chemical environment of a biopharmaceutical during SEC may alter the true state of aggregation of a biopharmaceutical sample, especially when reversible concentration-dependent aggregation is present. Hence, this article will highlight the new capabilities of AUC analysis and speculate on the important role AUC will play in the biopharmaceutical industry in terms of measuring aggregation.

ANALYTICAL ULTRACENTRIFUGES

The development and use of the analytical ultracentrifuge in studying the solution behavior of macromolecules dates back to the early twentieth century. This early work is highlighted by the pioneering efforts of Svedberg and his

Corresponding Author: Steven A. Berkowitz,
Department of Analytical Development, Biogen Idec Inc,
14 Cambridge Center, Cambridge, MA 02142.
Tel: (617) 678-2722; Fax: (617) 679-3476;
E-mail: steven.berkowitz@biogenidec.com

coworkers.[11] From this early period of time up until the 1970s AUC played an important role in formulating our knowledge of the biophysical properties of biopolymers (eg, protein, nucleic acid), supramolecular structures such as viruses and ribosomes, and synthetic polymers. Hence, for our early basic understanding of macromolecular structure and molecular biology we are significantly indebted to this important analytical instrument (details concerning the history of the analytical ultracentrifuge can be found in Lewis and Weiss,[12] Schachman,[13] Bowen,[14] Van Holde and Hansen,[15] Ranby,[16] and Righetti[17]). However, with the development of SEC (by Porath[18,19] in the late 1950s and early 1960s), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[20-22] and soft ionization mass spectroscopy techniques,[23,24] along with major shifts in research in the biological and biomedical sciences during the past several decades (owing to the developments of molecular biology), AUC virtually disappeared as a form of macromolecular analysis. But renewed interest in understanding protein-protein interactions and the realization of the need to have alternate analytical techniques to characterize the homogeneity of and level of aggregation in biopharmaceutical solutions has led to the modernization of and renewed interest in AUC.[13,25-34] In addition, advancements in computer technology, which have brought low-cost computer power to the scientist's desktop, have led to the development of several new and very useful AUC data analysis computer programs.[35-41] These programs have significantly improved the amount and quality of information that can now be extracted from AUC data. During this reemergence of AUC, several books and articles have appeared discussing its new benefits and capabilities.[37,42-50] More recent advances in AUC analysis[7,8,51-66] have brought even greater capability, so that AUC analysis can better characterize and quantify the homogeneity and biophysical solution properties of biopolymers.

AGGREGATION OF PROTEIN BIOPHARMACEUTICALS

A unique characteristic of proteins is their ability to aggregate with themselves (self-association), other proteins, or other chemical compounds (hetero-association) in specific ways to create a multitude of stable and dynamic biological structures. These specific spontaneous associations or aggregations of proteins (to form functional biological structures) are frequently referred to as self-assembly and can be reversible or irreversible. During the biosynthesis of proteins, and their existence within or outside the cell, opportunities exist for their misfolding, denaturation, and chemical (enzymatic and nonenzymatic) modification. These events can lead to unwanted nonspecific protein aggregation[67-70] (which predominately results from the weak forces that hold a protein structure together, the dynamic nature of a protein's struc-

ture,[71] and the reactivity of some of its chemical constituents[67,72,73]). In the case of biopharmaceuticals, the additional sources of physical handling, processing, and exposure of proteins to an array of different physical and chemical environments offer more opportunities for altering proteins' structure that may further enhance their ability to aggregate. If the production of these unwanted aggregates is not minimized and they are not removed, they will become part of the finished biopharmaceutical drug product and may alter the activity of the biopharmaceutical and cause adverse biological effects when administered to a patient (eg, immunogenicity).[74-76] Even the simple process of concentrating a biopharmaceutical can cause aggregation by driving specific and nonspecific interactions through mass action.[77] Although such aggregation can be reversible, the kinetics of the disassociation process may be very slow.[78] When these aggregated biopharmaceuticals are dosed into a patient, the slow disassociation process may be further altered because of the unique physical and chemical environment found in vivo, which can produce molecular crowding effects[33,79-82] (resulting from the very high macromolecular concentration environment of the fluids in the circulatory system and in the liquids found within and between cells) and charge effects (resulting from changes in ionic strength and pH). Hence, the simple and rapid disassociation of these types of aggregates from drug dilution during its administration may not mirror in vitro observations. As a result, the average lifetime of the reversible concentration-dependent aggregate within a patient could be increased significantly. Such behavior may lead to problems.

In characterizing the aggregation of a biopharmaceutical the initial amount of aggregation, the initial distribution of aggregate sizes (which describes the amount of material present with a specific number and arrangement of repeating monomer units), and changes in both these parameters with time, formulation matrix, and storage conditions is of great important. In particular, information concerning aggregation size appears to play an important role in protein immunogenicity.[83-86] Hence, even when the total amount of aggregation remains unchanged, changes in the size distribution of the aggregated biopharmaceutical still need to be carefully monitored.

SIZE-EXCLUSION CHROMATOGRAPHY

The positive attributes of SEC, mentioned in the introduction, have made SEC the biopharmaceutical industry's method of choice for detecting and quantifying the aggregation of biopharmaceuticals. Nevertheless, these highly favorable attributes must be weighted against the following issues, some of which have already been briefly mentioned:

1. SEC analysis is frequently conducted using a mobile phase that is significantly different from the buffer

matrix of the biopharmaceutical sample being studied. (In some cases, mobile phases may even contain organic solvents.) Such differences can alter the amount and the size distribution of the aggregation initially in a sample. This difference between a sample's matrix and the SEC mobile phase occurs for the following reasons:

○ The suppression of interactions of the biopharmaceuticals with the SEC chromatography material.

○ The need to run many samples in different buffer matrices in a short time. In this situation the use of a different SEC mobile phase to match each sample matrix buffer is impractical because of the large amount of time and labor it would require to re-equilibrate the HPLC system and, more importantly, may not be feasible because of interactions of the biopharmaceutical with the SEC chromatography material.

○ The lack of knowledge by the investigator of the possible changes that may occur to a sample's aggregation level and its distribution of sizes when the chemical environment of a sample is changed during SEC.

2. Sample preparation steps that include sample dilution and sample clarification are typically required before injecting a biopharmaceutical on an SEC column. Such sample treatments can alter the amount and the size distribution of aggregation initially in a sample. In the case of sample dilution, the ability to detect reversible concentration-dependent aggregation is significantly reduced. In the case of clarifying SEC samples (especially when membrane filters are used to prevent column fouling), the removal or selective removal of specific biopharmaceutical species (eg, aggregated material) may occur.

3. SEC columns and column hardware can frequently require conditioning before actual testing (using either the biopharmaceutical itself or another general protein, eg, bovine serum albumin) to eliminate the interaction of the biopharmaceutical with active sites in the SEC column or on the HPLC hardware. The masking of these active sites (which can vary from one lot of SEC material to another) is of critical importance in avoiding the total or selective removal of material, such as aggregates, from the injected biopharmaceutical sample. However, once an SEC column is conditioned, the secondary issue of the stability of this conditioning becomes another point of concern.

4. The need to have other reliable and orthogonal methods to confirm (qualified or validated) the accuracy of the amount of aggregation and its distribution of aggregate sizes measured by SEC.

5. The separation range of SEC columns may not be large enough to resolve aggregated material from the biopharmaceutical monomer or may provide limited information about the size distribution of the aggregates present. This problem arises when the pore size range in commercially available SEC chromatography material is too small in comparison to the actual size of the biopharmaceutical monomer and/or its aggregates.

ANALYTICAL ULTRACENTRIFUGATION, "BOUNDARY" SEDIMENTATION VELOCITY

AUC experiments typically fall into 2 categories: sedimentation velocity or sedimentation equilibrium. This article focuses on only sedimentation velocity. Discussion of AUC and sedimentation equilibrium can be found elsewhere.[59,87] Nevertheless, it should noted that in many cases the application of both types of sedimentation experiments can be very helpful in gaining an understanding of the biophysical solution properties of a protein system.

In conducting classical "boundary" sedimentation velocity experiments, a double sector-shaped centerpiece is commonly used. The first sector is filled with a sample solution, while the second is filled with a reference solution. (For UV detection it is possible to eliminate the reference solution, allowing the reference sector to be used as a second sample.[88] As a result, the number of samples that can be handled in one sedimentation velocity run can be doubled.) Acceleration of this cell to an appropriate speed and the acquisition of concentration data as a function of radius, r, at different times, t, yield a series of concentration profiles, $s(r, t)$, that reveals the complete migration pattern of all the different macromolecular species in a given sample. A theoretical example displaying a few of these concentration profiles that would be obtained for a monodisperse sample during a sedimentation velocity experiment is shown in Figure 1. In this case, each concentration profile consists of a region that displays a single sigmoidal-shaped curve that is called the boundary and a flat region at a greater radial distance from the boundary that is called the plateau. Concentration profiles obtained at later times show progressive boundary broadening and a reduction in the height of the plateau. The reduction in the plateau height is due to the sector shape of the cell (required to avoid convection resulting from the radial nature of the centrifugal force field[89]), while the sigmoidal-shape boundary and its broadening is due to diffusion. In 1929, Lamm[90] developed the partial differential equation that enables calculation of the radial-concentration profile and the change in this concentration profile with time, $s(r, t)$, for a monodisperse sample in a centrifugal field in a sector-shaped cell, using the sedimentation and diffusion coefficients of the material being studied. Unfortunately, this equation has no known general analytical solution, except
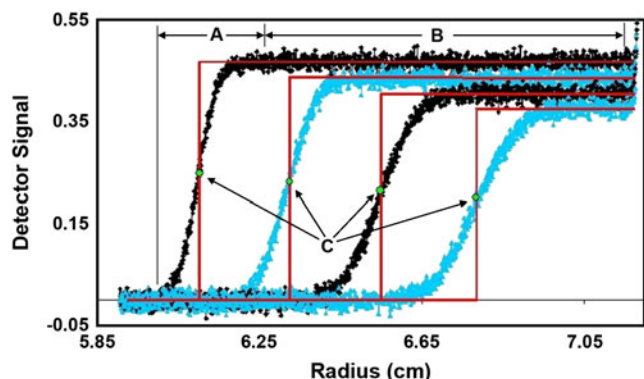
Figure 1. Theoretically calculated concentration profiles, s(r, t), generated for a simulated sedimentation velocity run on a sample containing only 1 molecular component with a molecular weight of 150 kDa and a sedimentation coefficient value of 6.5 S. The area in the first concentration profile, labeled A, corresponds to the region in the concentration profile called the boundary, while the area labeled B corresponds to the region in the concentration profile called the plateau. All analytical ultracentrifugation concentration profiles were generated using SEDFIT with a time interval of 2000 seconds. The profiles were calculated for a sedimentation velocity experiment conducted at a speed of 50 000 rpm, at 20°C, with a noise level of 0.01. The corresponding concentration profiles that would be obtained in the absence of diffusion are indicated by the traces (with no noise) in red. Points labeled C correspond to the radial position in the boundary region equal to the half-height of the plateau region for each concentration profile. These points would be used to calculate the sedimentation coefficient of the sample (Figure 2).
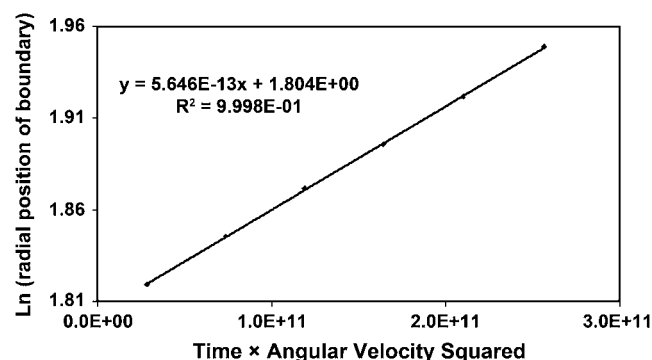

Figure 2. A typical plot of the natural log of the radial boundary position (which usually corresponds to the radial position in the boundary that equals the half-height of the plateau region) of each concentration profile as a function of time, in seconds, multiplied by the angular velocity, radian per second, squared. The slope of this plot yields the sedimentation coefficient of the molecular species in the sample. If more than one species were in the sample, the sedimentation coefficient would correspond to a weight-average sedimentation coefficient, S_w. Technically, for the slope to accurately correspond to the sedimentation coefficient of the sample, the radial position of the boundary used should be equal to the square root of the second moment of the boundary.[93] However, in many cases the difference between the radial position corresponding to the square root of the second moment of the boundary and the radial position in the boundary that corresponds to the half-height of the plateau is too small to be significant.

for some very special cases.[91] As a result, until recently, relatively little general use has been made of this equation for routine sedimentation velocity analysis. For many years, sedimentation velocity experiments were simply conducted and analyzed to obtain the sedimentation coefficient of a sample,[92] as shown in Figure 2. In addition, preliminary qualitative assessment of a sample's high level of homogeneity was frequently demonstrated by sedimentation velocity experiments that showed the visual presence of only a single migrating boundary (Figure 3C shows that this boundary is not a rigorous criterion for assessing a sample's homogeneity).

MODERN SEDIMENTATION VELOCITY ANALYSIS

It is important to note that sedimentation velocity experiments in an analytical ultracentrifuge are conducted under very well controlled experimental conditions (temperature, centrifugal field (rotor speed), and defined and fixed geometry), with minimal sample contact with surfaces and with no sample manipulation or sample preparation steps other than possibly sample dilution. A sample can be taken directly from the sample vial and placed into the assembled AUC cell. (This sample management approach is particularly applicable when UV detection is used. However, the

use of refractometric detection [interference optics] frequently requires some minor sample treatment; see the brief discussion on sample treatment in the Advantages of Using AUC to Help Assess Biopharmaceutical Aggregation section.) Furthermore, the underlying physics and equations that explain the movement of the molecules in these experiments are well understood. All of this, combined with the automated data acquisition capability of the modern analytical ultracentrifuge and the recent availability of powerful desktop computers, has provided the opportunity for several investigators to develop advanced numerical analysis computer programs for the analysis of sedimentation velocity experiments.[7,8,35,36,38,40,47,48,51,54,58] These software programs now offer significant capability to extract information from sedimentation velocity experiments that was either not feasible or far too difficult to do in the past. In this article, we will focus on one of the more recent AUC computer programs called SEDFIT, developed by Schuck and coworkers.[8,51,54] Recently Schuck and coworkers have developed a second computer program for AUC analysis called SEDPHAT.[58,59] This second program offers significantly more advanced capabilities for analyzing AUC data by enabling global analysis procedures to be applied to several different types of experiments to extract more detailed and reliable information about a particular macromolecular system. Discussion of this program,
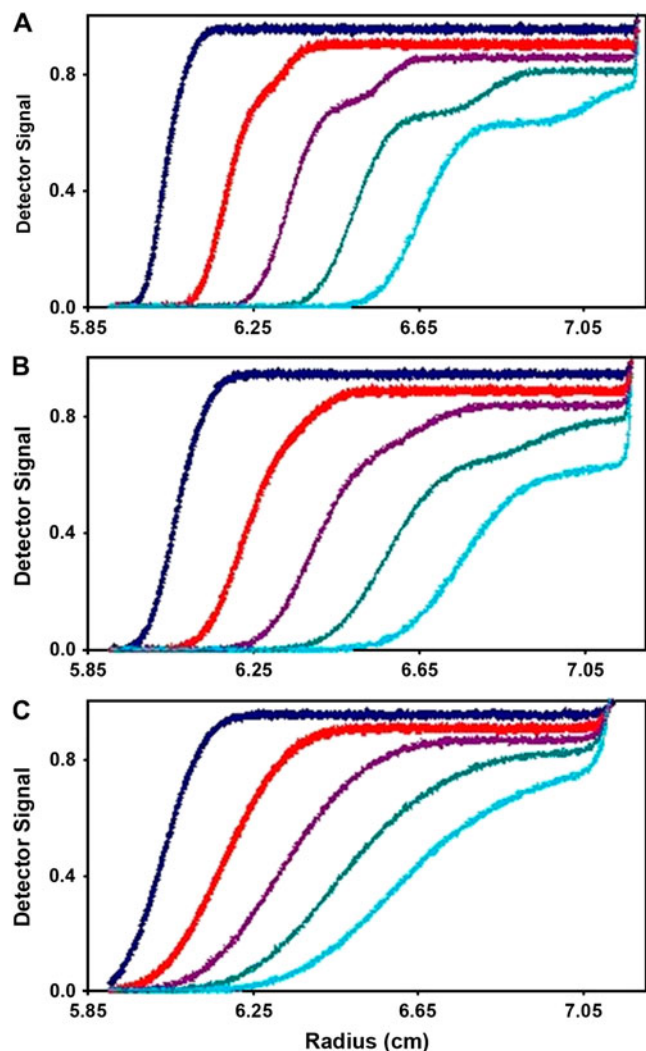
Figure 3. Simulated sedimentation velocity experiments that illustrate the effect of molecular size on the ability to visually detect a secondary boundary when a sample contains only a monomer and dimer in the fixed ratio of 80:20. Calculations were conducted for monomer-dimer pairs having the following molecular weights and sedimentation coefficients: (A) 150 kDa, 6.0 S monomer and 300 kDa, 9.0 S dimer; (B) 75 kDa, 4 S monomer and 150 kDa, 6.0 S dimer; and (C) 25 kDa, 2.0 S monomer and 50 kDa, 3.0 S dimer. All theoretically calculated sedimentation velocity concentration profiles, s(r, t), were generated using SEDFIT. Analytical ultracentrifugation experimental conditions held constant for all calculations were speed = 50 000 rpm, temperature = 20°C, and experimental noise level = 0.005. Data acquisition time intervals varied for each experiment.

however, is beyond the scope of this article. (Readers interested in this newer AUC program should consult references provided in this article and Peter Schuck's Web site [http://analyticalultracentrifugation.com/sedphat/sedphat.htm] for more information.)

One of the important capabilities of SEDFIT is its ability to extract information concerning the amount and the distribution of aggregate sizes in a sample. The brief discus-

sion provided below attempts to outline some of the very basic approaches used in this computer program. However, the reader is advised to consult recent reviews by Balbo and Schuck,[94,95] Dam and Schuck,[8] and Lebowitz et al,[7] and the original publications of Schuck and his collaborators,[51-63] to acquire a more complete understanding of the operation, capabilities, and limitations of this AUC analysis program.

Returning to the data in Figure 1, for a monodisperse sample, and assuming for a moment that the concentration profiles, s(r, t), in this figure are actually experimental data, an approach could be taken to numerically solve the Lamm equation, f(s, D, r, t). This could be achieved via numerical procedures that conduct a search for the sedimentation, s, and diffusion, D, coefficients (within a realistic constrained range of s and D values) that generate theoretical concentration profiles that best match the entire set of experimental concentration profiles, s(r, t), collected during an AUC experiment. If this numerical analysis is conducted under appropriate statistical criteria of goodness-of-fit, which typical involves minimizing the sum of the squares of deviations between the experimental and theoretical data, as shown in Equation 1 below, useful molecular information concerning the sedimentation and diffusion coefficients and molecular weight of the single molecular component in the sample could be obtained.

$$\sum[s(r, t) - f(s, D, r, t)]^2 = \sum[\text{experiment data} - \text{theoretical data}]^2$$

The real utility of this approach, however, is in applying it to the more general problem of determining the level of homogeneity or the actual polydispersity of a real sample. In the simplest case of a polydisperse sample, where only 2 components are present, the numerical analysis would require the summation of 2 separate Lamm equation solutions to match the experimental data, s(r, t): one for component 1, $f(s_1, D_1, r, t)$, and one for component 2, $f(s_2, D_2, r, t)$. Each solution would then be multiplied by the actual concentration of that component, c_1 and c_2, and summed as shown by Equation 2:

$$s(r, t) = c_1 f(s_1, D_1, r, t) + c_2 f(s_2, D_2, r, t)$$

Hence, to solve this problem, as outlined in Equation 1, it is necessary to find in addition to the best value for the molecular parameters s_1, s_2, D_1, and D_2, the best concentration value for component 1, c_1, and component 2, c_2. This approach can be further expanded to n components, as indicated by Equation 3:

$$s(r, t) = \sum[c_n f(s_n, D_n, r, t)]$$

If the number of components considered is large, and if the concentration and the diffusion coefficient can be expressed

as a function of the sedimentation coefficient, s, the number of components present could be considered a continuous function of s, and Equation 3 can be represented by the integral equation shown by Equation 4:

$$s(r, t) = \int [c(s)\, f(s, D(s), r, t)]\, ds$$

Evaluation and plotting of the differential sedimentation distribution value, c(s) (which corresponds to the amount of material in a sample having a sedimentation coefficient between s and s + ds), vs s yields the distribution of sedimentation coefficients for the sample being analyzed. This plot gives information very similar to that provided by an SEC chromatogram, as shown in Figure 4. From the c(s) vs s plot, the homogeneity of a sample can be assessed and the amount of each component determined by appropriate integration. It should be noted that in some cases the presence of different components in a sample can be seen in AUC concentration profiles, s(r, t), as separate resolved boundaries or as a sloping plateau. The occurrence of these visual clues, however, is very dependent on the differences in the sedimentation and diffusion coefficients between the components present and the relative number and amounts of each component (Figures 3 and 5).

The numerical approach used in SEDFIT to find the simplest and most accurate values for c(s) that will solve Equation 4, again with the same basic method outlined by Equation 1, is based on 3 key concepts: (1) the diffusion coefficient for each component can be calculated in terms of its sedimentation coefficient, D(s); (2) steps can be taken to avoid the overfitting or underfitting of the data; and (3) all molecular components in a sample must migrate independently of each other. For concept 1, SEDFIT uses a function derived by combining the Stokes-Einstein and Svedberg equations (Equation 25 in the paper by Dam and Schuck[8]). The success of these calculations depends on finding the best weight-average value for the translational frictional coefficient ratio, $(f/f_o)_w$, for the entire sample. The ratio f/f_o corresponds to the ratio of the translational frictional coefficient of a molecule relative to its theoretical translational frictional coefficient when the same molecule takes on the shape of a sphere of the same density.[8] It should be noted that the validity of applying the same $(f/f_o)_w$ value to all molecular components in a given sample has limitations. A discussion of this topic can be found in works by Dam and Schuck,[8] Philo,[10] Schuck,[51,54] and Schuck et al.[57] In the case of concept 2, which deals with the quality of the numerical fit, asking for the highest fidelity
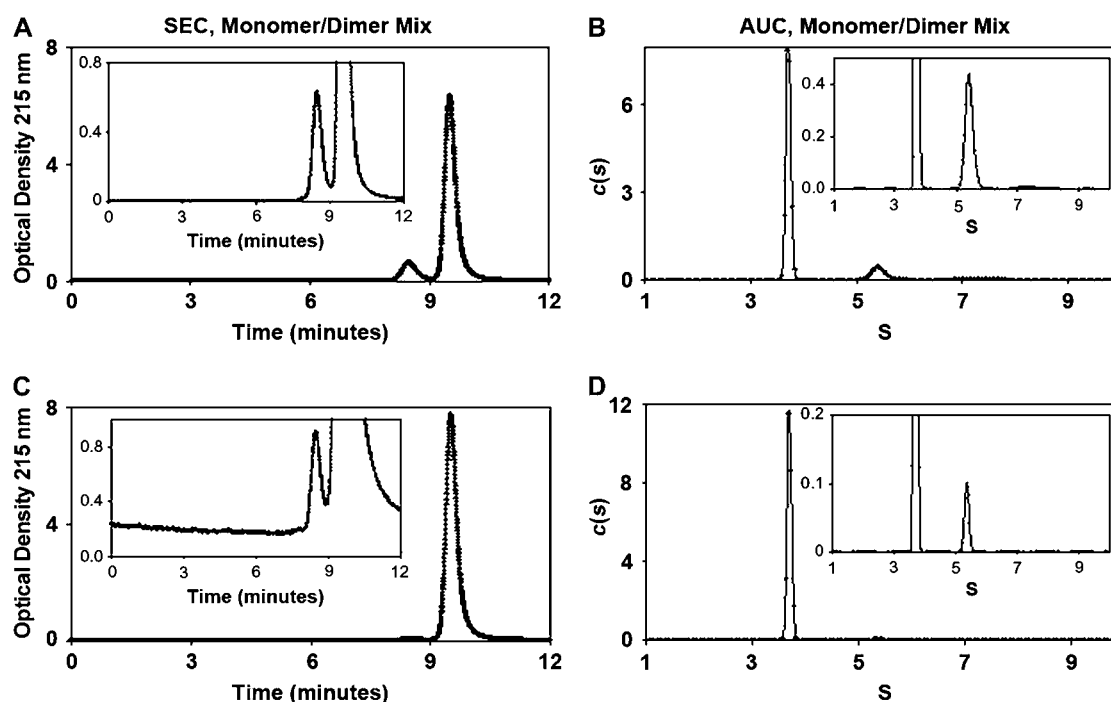

Figure 4. Comparison of size-exclusion chromatography (SEC) and analytical ultracentrifugation (AUC) results for 2 biopharmaceutical samples containing a total aggregation level of ~1% and 12% (predominately dimer). Quantitative results obtained for these 2 experiments and a third experiment in which the level of aggregation was ~3% are shown in Table 1. (A) SEC chromatogram for the sample with 12% aggregation; (B) plot of the distribution of sedimentation coefficients (c(s) vs s, where s is plotted in Svedberg units, S) generated from an AUC experiment on the same sample used in part A using SEDFIT; (C) SEC chromatogram of a sample containing 1% aggregation; and (D) plot of the distribution of sedimentation coefficients (c(s) vs s, where s is plotted in Svedberg units, S) generated from an AUC experiment on the same sample used in part C using SEDFIT. (All inserts shown in each graph correspond to an expanded view of the same graph along the Y-axis.)
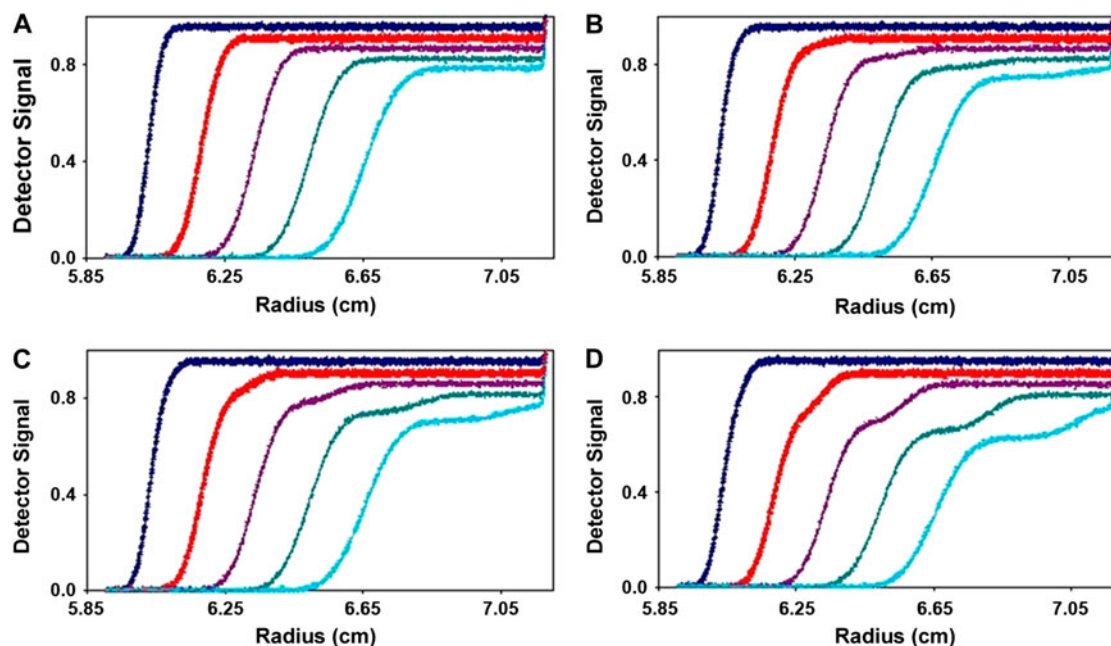
Figure 5. Simulated sedimentation velocity experiments that illustrate the effect of having different ratios of 2 components on the ability to visually detect a secondary boundary. All calculations were conducted for theoretical samples composed of different ratios of a 150-kDa monomer having a sedimentation coefficient of 6.0 S, and a 300-kDa dimer aggregate having a sedimentation coefficient of 9.0 S, for samples having the following ratio of monomer:dimer: (A) 100:0, (B) 95:5, (C) 90:10, and (D) 80:20. All theoretically calculated sedimentation velocity concentration profiles, s(r, t), were generated using SEDFIT. Analytical ultracentrifugation experimental conditions held constant for all calculations were speed = 50 000 rpm, temperature = 20°C, experimental noise level = 0.005, and data acquisition time interval = 2000 seconds.

to minimize the difference between the experimental data (with its accompanying noise) and the theoretical data can create artifacts (because the optimization search routine will force the theoretical data to match the experimental noise). However, too much of a reduction in the quality of the fit between the experimental and theoretical data will also give misleading information by failing to appropriately match the "real" data. Hence, the task of finding just the right balance in the quality of fit is important. In SEDFIT, the use of regularization (a mathematical tool that can control the fitting process) with a confidence level typically set at 0.7 is commonly recommended to achieve this goal[8] (the operator can adjust the confidence level and therefore experiment with the level of regularization applied). For concept 3, all species must display ideal thermodynamic and hydrodynamic behavior. Such behavior includes the absence of excluded volume and electrostatic effects, and interactions/aggregation or changes in aggregation within the time scale of the sedimentation experiment. As a result, the absence of such non-ideality effects needs to be assessed. In most cases non-ideality can be avoided by using low sample concentrations (roughly 1 mg/mL or less) and making sure the salt concentration is high enough (roughly 0.05M or greater).

The numerical approach used by Schuck and coworkers to solve Equation 4 involves a collection of mathematical tools that include finite element analysis to numerically solve the Lamm equations guided by nonlinear least squares optimization calculations with appropriate regularization. These calculations, which are typically conducted to find the best $(f/f_0)_w$ within a specified (constrained) range and resolution of s values, may also involve the optimization of additional experimental parameters (initially set by the operator) at the same time. These other parameters include the radial positions of the sample solution meniscus and cell bottom. This modern approach of direct boundary modeling of the entire sedimentation velocity process requires far more data than did the older approach of sedimentation velocity analysis, which required only a few concentration profiles, as shown in Figure 6.

The success and utility of this modern approach for extracting information from sedimentation velocity data are illustrated in the SEC vs AUC section, with real examples from my experience in using SEDFIT on a range of biopharmaceuticals. However, before discussing these examples it should be noted that the collection of constantly changing concentration profiles acquired during a sedimentation velocity experiment is a very rich and unique source of information. Hence, the fitting of all the experimental data within a realistic limited range of s values and resolution of the s step size (set by the operator) puts significant constraints on which models can be used to adequately fit the data. The more the fitting process can be constrained with information, the more accurate will be the information generated by the
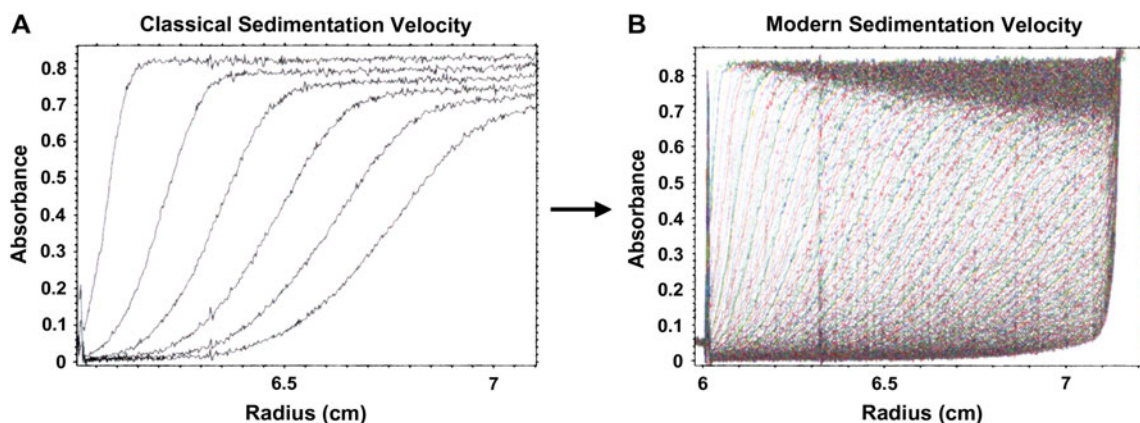
Figure 6. (A) Classical amount of analytical ultracentrifugation (AUC) data required for determining the sedimentation coefficient of a sample from a sedimentation velocity experiment in comparison to (B) the modern approach of AUC data analysis, using SEDFIT, which requires large amounts of AUC data to accurately calculate the distribution of sedimentation coefficients, c(s), of a sample. The latter approach is now capable of extracting from a sedimentation velocity experiment the number and amounts of different species that are in the sample even when individual boundaries for each component are not visible (eg, Figure 3C).

mathematical tools in SEDFIT, which searches out the best and simplest answer to minimize Equation 1.

SEC VS AUC

When the form of sedimentation velocity analysis discussed in Modern Sedimentation Velocity Analysis is applied to the assessment of biopharmaceutical aggregation, the sensitivity (in terms of limit of quantification), precision (in terms of repeatability, intermediate precision, and reproducibility), and accuracy of the results obtained by SEDFIT are important issues. Data shown in Figure 4 compare the SEC elution profile with the calculated distribution of sedimentation coefficients obtained by AUC analysis conducted on the same samples, where the aggregation content varied from 1% to 12%. Appropriate integration of the resulting peak areas from these and other plots produced the aggregation information in Table 1. These results indicate a good

Table 1. Summary of SEC and AUC Analysis on 3 Different Protein Samples Containing Different Amounts of Aggregation*

	SEC			
Sample	**% Monomer**	**% Dimer**	**% HMW**	**% Total Agg**
Sample 1	88.2	11.7	0.2	12.0
Sample 2	96.6	3.4†		3.4
Sample 3	98.9	1.1†		1.1
	AUC			
Sample	**% Monomer**	**% Dimer**	**% HMW**	**% Total Agg**
Sample 1	87.5	11.9	0.6	12.5
Sample 2	96.6	3.4		3.4
Sample 3	98.8	1.2		1.2
	% Difference (between SEC and AUC total aggregation)‡			
Sample 1	4.7			
Sample 2	1.4			
Sample 3	12.1			

*SEC indicates size-exclusion chromatography; HMW, high–molecular weight aggregates; Agg, aggregation; and AUC, analytical ultracentrifugation, and ABS, absolute value.
†Value corresponds to % Dimer + % HMW.
‡% Difference = 100 (ABS|SEC(n) − AUC(n)|/((SEC(n) + AUC(n))/2)), where SEC(n) and AUC(n) equal % Total Aggregation for sample n as measured by SEC and AUC, respectively.

agreement between AUC and SEC over the aggregation range investigated. Hence, good sensitivity using AUC can be achieved down to aggregation levels of ~1% (using UV detection with absorbance loads in the range of ~0.4-1.2). In fact, Dam and Schuck state that their computer simulation work has shown that using "a set of profiles covering the complete sedimentation process at a signal-to-noise ratio of 200:1 (which can be readily achieved, eg, at a loading concentration of 0.3-0.4 mg/mL of protein in the interference optics), minor peaks consisting of 0.2% of the total protein concentration can be readily detected."[8] However, it is the author's experience that efforts to quantify components below ~1% put considerable demands on the data and the data analysis process.

Data shown in Figure 7 provide additional information on the intermediate precision and comparability of SEC and AUC for a more highly aggregated and more complex sample. Quantitative SEC and AUC results obtained for this sample on 3 different days are shown in Table 2. Again, results show good agreement and good precision. When a consistent level of agreement, as indicated in Tables 1 and 2, is obtained between these 2 orthogonal methods, significant confidence in the accuracy of SEC aggregation information is generated. In general, a level of agreement achieved in terms of total aggregation between AUC and SEC (expressed as percentage difference; Tables 1 and 2) that is about ±10% to 20% or better is a tentative criterion used in our lab to indicate that SEC is providing adequate accuracy. It should be noted that this criterion is typically applied when the total aggregation level determined by AUC is 1% or higher. Nevertheless, the real clear-cut benefit of AUC in assessing SEC methods is in cases where very large and consistent differences exist between these 2 methods, as illustrated in Figures 8 and 9. Such situations typically highlight the utility of AUC in uncovering SEC problems or in revealing solution properties of biopharmaceuticals that SEC missed.

Results shown in Figures 4 and 7 also illustrate the ability of AUC to provide better resolution than SEC. This point is particularly highlighted in Figure 7 for the high–molecular
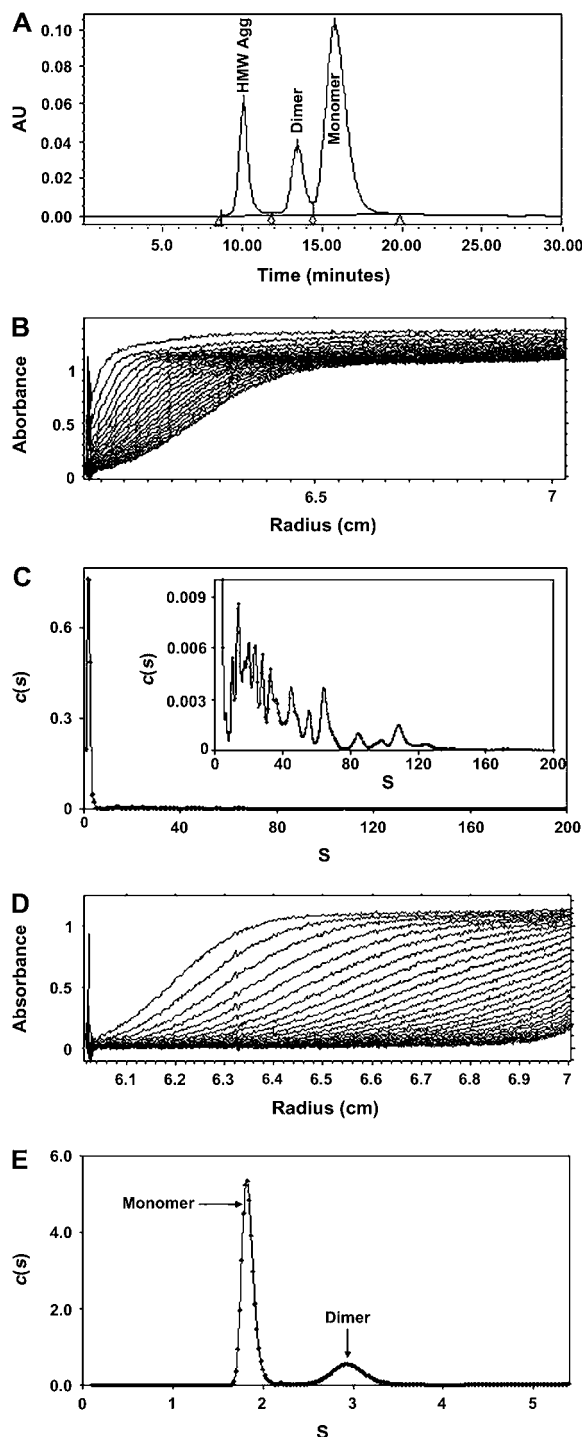
Figure 7. Comparison of size-exclusion chromatography (SEC) and analytical ultracentrifugation (AUC) results for a biopharmaceutical containing a high and more complex amount of aggregation. (A) SEC chromatogram of a sample showing the presence of dimer plus high–molecular weight (HMW) species. (B) First 25 raw AUC data concentration profiles from a sedimentation velocity run on the same sample used in part A. (C) Plot of the distribution of sedimentation coefficients (c(s) vs s, where s is plotted in Svedberg units, S) calculated from the concentration profiles shown in part B using SEDFIT. AUC results clearly show the high level of heterogeneity of the aggregated material in this sample in comparison to the single HMW peak seen in the SEC profile shown in part A. (D) The remaining AUC concentration profiles (profiles 25-125; every 5th profile is displayed) from the same sedimentation velocity experiment used in part B after all of the HMW material had migrated to the bottom of the AUC cell. (E) Plot of the distribution of sedimentation coefficients (c(s) vs s, where s is plotted in Svedberg units, S) calculated from the concentration profiles shown in part D using SEDFIT; this provides information on the amount of monomer and dimer material in this biopharmaceutical sample. The overall agreement obtained between SEC and AUC results for this sample are summarized in Table 2. Agg indicates aggregation.

Table 2. Summary of SEC and AUC Analysis on a Protein Sample Containing Aggregated Material on 3 Different Days*

		SEC		
Sample	**% Monomer**	**% Dimer**	**% HMW**	**% Total Agg**
Sample 1	67.3	14.9	17.8	32.7
Sample 2	67.0	15.1	17.9	33.0
Sample 3	66.9	15.2	18.0	33.2
Average	67.1	15.1	17.9	33.0
SD	0.2	0.2	0.1	0.3
		AUC		
Sample	**% Monomer**	**% Dimer**	**% HMW**	**% Total Agg**
Sample 1	65.7	16.0	18.3	34.3
Sample 2	65.1	18.1	16.8	32.9
Sample 3	65.2	18.3	16.5	34.8
Average	65.3	17.5	17.2	34.7
SD	0.3	1.3	1.0	0.3
% Difference (between SEC and AUC total aggregation)† = 5.0%				

*SEC indicates size-exclusion chromatography; HMW, high–molecular weight aggregates; Agg, aggregation; and AUC, analytical ultracentrifugation.

†% Difference = 100 (ABS|SEC − AUC|/((SEC + AUC)/2)), where SEC and AUC are the average Total Aggregation measured by SEC and AUC, respectively.

weight (HMW) material. In the case of SEC, the HMW material piles up into a single excluded volume peak. In the case of AUC, this material is resolved to reveal a very heterogeneous collection of molecular species. Such resolution makes it possible to monitor changes in the HMW material that could not be detected by SEC. This higher resolution and enormous dynamic size range of AUC analysis is further demonstrated in Figure 10. In this example, AUC is used to assess the homogeneity of an adenovirus gene therapy biopharmaceutical. Because of the large size of the monomer unit (which has a diameter of ~900 Å) and its aggregates, SEC cannot be used to monitor the aggregation and the distribution of the aggregate sizes in samples of this biopharmaceutical. However, AUC analysis conducted using the interference optics (which minimizes problems associated with differences in the response factor for different molecular components in this sample) at 3000 rpm provides the results shown in Figure 10. In the case of samples A and B, the concentration profiles clearly reveal the presence of 2 boundaries. The small, slow-moving boundary seen (toward the end of the run) in both preparations corresponds to the peak labeled "empty capsid" in the c(s) vs s plot. This material is a common assembly impurity product made during normal adenovirus production[96] that is not removed during large-scale virus purification.[97] The much larger and faster-moving boundary, seen in both cases, corresponds to the intact virus monomer and is labeled "monomer" in the c(s) vs s plot. All material mov-

ing faster than the virus monomer represents other virus species that include adenovirus aggregates. Close inspection of the plateau region of the concentration profiles obtained for both virus preparations reveals that sample B contains a much higher level of faster-sedimenting material than sample A does. This difference can be seen by the greater sloping of the plateau region in concentration profiles for sample B than in concentration profiles for sample A. This visual observation is confirmed by quantitative AUC analysis with SEDFIT, where the apparent aggregation value for sample A was determined to be ~4%, while the apparent aggregation value for sample B was determined to be ~23% (Berkowitz and Philo, unpublished data, August 15, 2006).

Data shown in Figure 8 illustrate an issue frequently encountered in SEC: variability in the amount of SEC aggregation determined for the same sample as a result of using different SEC mobile phases. In this case, 3 different mobile phases were investigated, yielding the SEC aggregation results shown in Figures 8A through 8C. However, the good agreement in the total amount of aggregation determined by AUC analysis in Figure 8D (on the same sample in its formulation buffer) with the SEC results obtained in Figure 8B indicates that buffer system 2 is the appropriate SEC mobile phase to use to obtain accurate aggregation for this protein.

Finally, AUC results in Figure 9 show the unique self-associating properties of a biopharmaceutical (in a specific
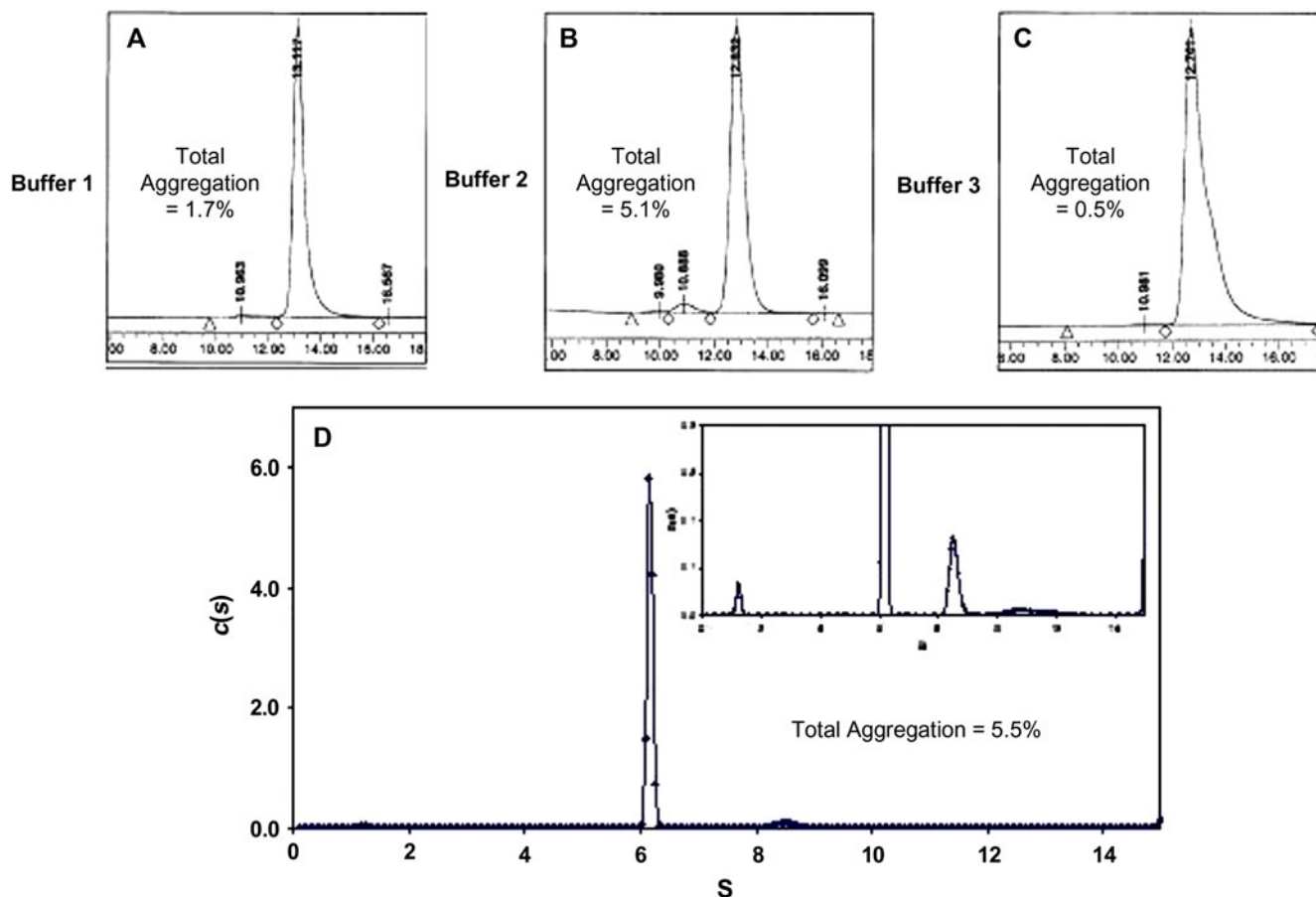
Figure 8. The use of sedimentation velocity to assess which size-exclusion chromatography (SEC) mobile phase is actually providing accurate information on the level of aggregated material in a biopharmaceutical sample. (A-C) Plots correspond to the SEC chromatograms obtained for the same biopharmaceutical sample using the same SEC column but different mobile phases (the y-axis corresponds to absorbance and the x-axis corresponds to time, minutes); (D) Plot of the distribution of sedimentation coefficients (c(s) vs s, where s is plotted in Svedberg units, S) obtained from the sedimentation velocity experiment on the same biopharmaceutical run in parts A through C using SEDFIT. (An analytical ultracentrifugation experiment was conducted in the formulation buffer of the biopharmaceutical.)

buffer) as a function of its concentration. At a concentration of 0.2 mg/mL, the sedimentation coefficient distribution of this biopharmaceutical shows a single major peak in addition to a much smaller but faster-migrating peak (Figure 9A). When the concentration of the biopharmaceutical is increased to 5.9 mg/mL, the faster-moving peak becomes the major peak and moves with a slightly higher sedimentation coefficient (Figure 9D). Such a behavior illustrates the concentration-dependent aggregation properties of this biopharmaceutical. This behavior is supported by simple model-free AUC analysis that yields the weight-average sedimentation coefficient (S_w) shown in Figure 9 for each sample concentration. In this analysis, the S_w values for this biopharmaceutical are observed to be an increasing function of concentration. This behavior is a classical diagnostic for concentration-dependent aggregation[98] and supports the results obtained by SEDFIT. In SEC analysis of this biopharmaceutical, this concentration-dependent behavior was missed because SEC results (data not shown) showed the

presence of only a single peak in all cases. The ability to detect such reversible chemical-reacting systems by AUC (even when the accuracy of the computed c(s) vs s plot is lacking, owing to the dynamic nature of the aggregation process relative to the time scale of the AUC experiment) can be important in biopharmaceutical development, specifically when the kinetics of disassociation for the aggregated biopharmaceutical in vivo (when administered to a patient) is very slow, as mentioned in the Aggregation of Protein Biopharmaceuticals section.

ADVANTAGES OF USING AUC TO HELP ASSESS BIOPHARMACEUTICAL AGGREGATION

Using AUC to assess the aggregation of a biopharmaceutical provides several distinct advantages over using SEC:

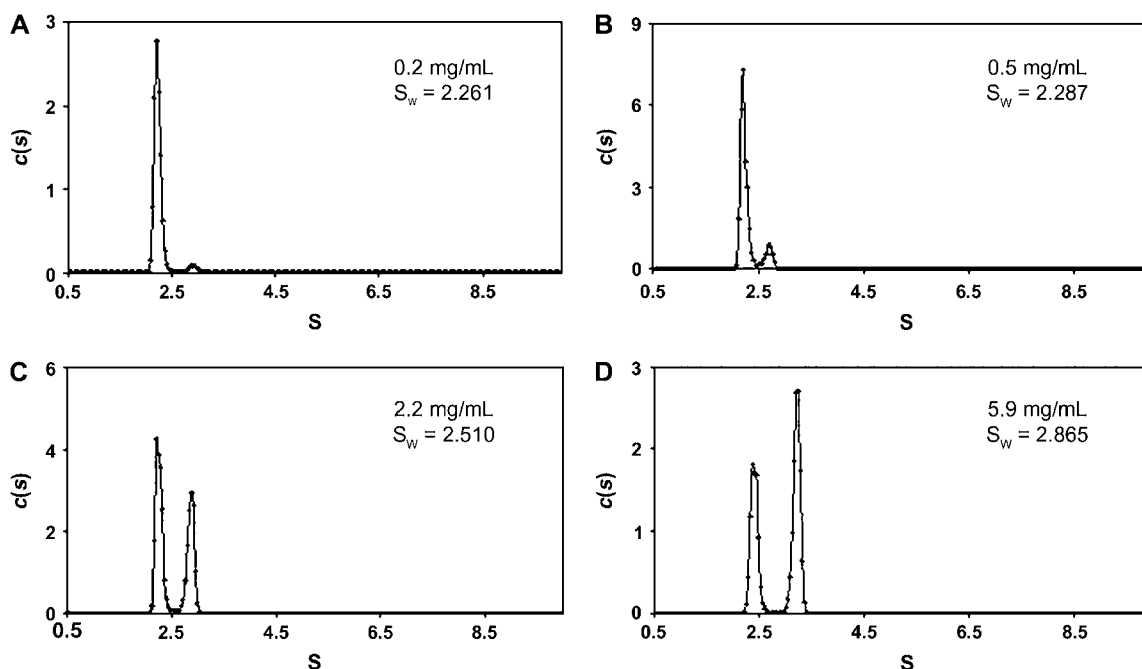1. The level of experimental method development required is minimal.

Figure 9. Reversible concentration-dependent aggregation properties of a biopharmaceutical detected using sedimentation velocity. Plots A-D correspond to the calculated distribution of sedimentation coefficients (c(s) vs s, where s is plotted in Svedberg units, S) obtained from sedimentation velocity experiments on the same biopharmaceutical run in its formulation buffer at different concentrations (indicated in each plot) using SEDFIT. S_w values (indicated in each plot) correspond to the weight-average sedimentation coefficient calculated for the entire sample.

2. No sample preparation is required, other than possibly sample dilution to investigate the effect of protein concentration on aggregation. However, when using an analytical ultracentrifuge's interference optical system (the Rayleigh interferometer,[99] which is a special and useful differential refractive index detector present on the AUC XL-I that enables very accurate concentration measurements to be made on biopharmaceuticals that lack chemical groups that absorb light, that generate variable light scattering background, or that are embedded in a buffer matrix that strongly adsorbs light), some simple steps (eg, sample dialysis) may be needed to match the sample and reference solution buffer matrix.[100] This sample preparation step is required to avoid artifacts from the differential sedimentation of buffer components due to differences in the buffer concentration between the reference and sample solutions.

3. Biopharmaceuticals can be run in its exact formulation buffer (within the limits of chemical stability of the centerpiece and windows of the AUC cell) and at the formulation concentration of the biopharmaceutical, as it exists in the vial. It should be noted, however, that although very high protein concentrations (eg, 40-50 mg/mL) can be analyzed in the analytical ultracentrifuge (especially when using the 3-mm centerpiece and the Rayleigh interferometer) the effect of non-ideal thermodynamic and hydrodynamic behavior can significantly limit the utility of the data obtained. Nevertheless, the ability to detect gross behavior changes and to conduct experiments at protein concentrations in the range of a few milligrams/milliliter and to possibly 10 mg/mL without encountering a significant impact from these non-ideality effects can provide qualitative insight as to what might be going on at much higher protein concentrations.

4. Several samples in different formulation buffers can be simply and accurately analyzed in the same AUC run. Typically, 7 to 8 samples can be analyzed in 1 AUC run using the 8-hole AN50Ti rotor (however, as mentioned earlier, under appropriate conditions sample throughput can be doubled[88]).

5. The amount of surface area that a sample encounters during AUC is minimal. The only material an AUC sample encounters is the sapphire or quartz window and the Epon charcoal-filled plastic or aluminum centerpiece.

6. AUC can be operated over a range of speeds (from ~2 K to 60 K rpm). Hence, the molecular weight and size range that it can characterize is enormous: from molecules of only a few hundred daltons[30] to particles that are almost a micron in size.[101,102] In some cases, AUC analysis methods have been developed
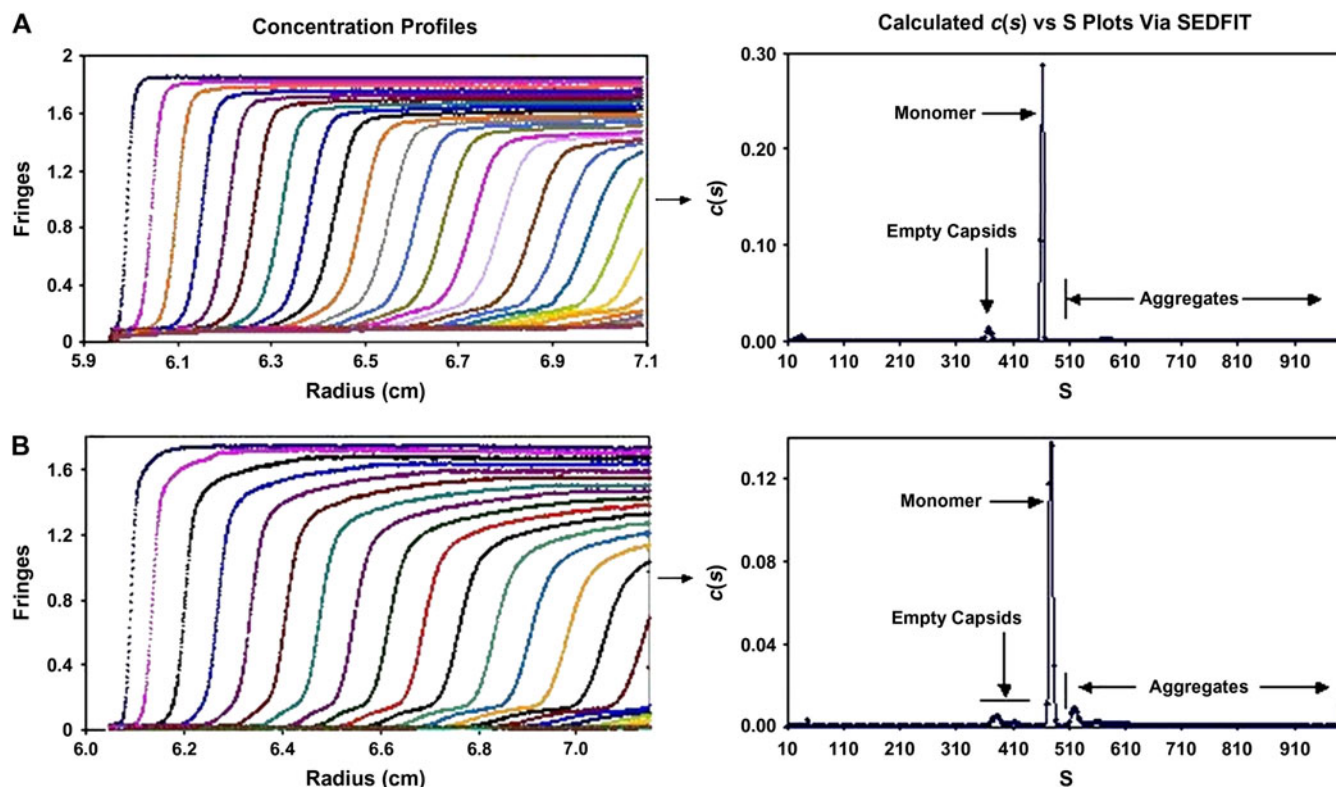
Figure 10. Characterization of 2 different adenovirus preparations using sedimentation velocity and data analysis with SEDFIT. Plots on the left side of this figure correspond to overlay of concentration profiles, s(r, t), acquired using the Rayleigh interferometer (corrected for radial and time invariant noise), while the graphs on the right correspond to distribution of sedimentation coefficients (c(s) vs s, where s is plotted in Svedberg units, S) calculated from the corresponding concentration profiles using SEDFIT. (A) Virus sample from a preparation containing only one type of empty capsids and a low amount of apparent aggregation; and (B) virus sample from a preparation containing more than one type of empty capsids and a much higher amount of apparent aggregation.

that allow the rotor's speed to be varied during a single run. Hence, a single sedimentation velocity run can analyze very polydisperse samples containing molecular species having sedimentation coefficient values from 1 or 2 S to 250 000 S.[101,102]

7. AUC can also provide the following additional information about a sample:

   ○ Energetic and stoichiometric information about molecular interactions
   ○ Detection of conformational changes
   ○ Extinction coefficients
   ○ Sample concentration
   ○ Buoyant density heterogeneity
   ○ Molecular shape
   ○ Solvation/hydration

CONCLUSIONS

As discussed in this article and other recent publications, analytical ultracentrifuges offer new, enhanced capabilities for assessing the aggregation and homogeneity of biophar-

maceuticals.[6-10,28-33,37,42-50,64,103] However, this is not to suggest that AUC should replace SEC. The processing of large numbers of samples on a daily basis and in a regulatory-compliant testing environment would present major challenges for AUC. Hence, at present, AUC will best service today's biopharmaceutical industry by supporting efforts in the development of accurate SEC methods and in the generation of important biophysical characterization information on potential biopharmaceuticals to help identify, design, and formulate new commercial drug products. Yet the task of implementing the widespread use of AUC analysis in the biopharmaceutical industry is stymied by the following 2 problems: (1) the relatively high cost of AUC instrumentation (especially for small biopharmaceutical companies), and (2) the small numbers of scientists who understand AUC analysis. The latter problem is the more challenging because for many years young scientists received little or no training in AUC analysis in academia. As a result, many biopharmaceutical scientists find the science (and mathematics) associated with AUC analysis to be foreign and intimidating. It is hoped that through courses, seminars, and workshops such as those offered by Schuck (at the National Institutes of Health, Bethesda, MD, http://dbeps.ors.od.nih.

gov./pbr_auc.htm), Demeler (at the University of Texas Health Science Center at San Antonio, http://www.cauma.uthscsa.edu/), Cole (at the University of Connecticut Biotechnology Center, http://www.biotech.uconn.edu/auf/), and Beckman Coulter Inc (Fullerton, CA, http://www.beckmancoulter.com), the sole manufacturer of the analytical ultracentrifuge, and through the published work of others, the more common use and acceptance of AUC analysis in the biopharmaceutical industry will be realized. As Howard Schachman[13] would say, "We can hardly wait!"

REFERENCES

1. Kamberi M, Chung P, DeVas R, et al. Analysis of non-covalent aggregation of synthetic hPTH (1-34) by size-exclusion chromatography and the importance of suppression of non-specific interactions for precise quantitation. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2004;810:151-155.

2. Clodfelter DK, Nussbaum MA, Reilly J. Comparison of free solution capillary electrophoresis and size exclusion chromatography for quantitating non-covalent aggregation of an acylated peptide. *J Pharm Biomed Anal*. 1999;19:763-775.

3. Link GW, Jr, Keller PL, Stout RW, Banes AJ. Effect of solution used for storage of size-exclusion columns on the subsequent chromatography of peptides and proteins. *J Chromatogr*. 1985;331:253-264.

4. Higley TJ, Cornell R. Improve recovery and reproducibility with pretreatment of analytical TSK-gel G3000SWxl size-exclusion columns. In: *Supplement to LC/GC North America: The Application Notebook*. Cleveland, OH: Advanstar Communications; 2004.

5. Chirino AJ, Mire-Sluis A. Characterizing biological products and assessing comparability following manufacturing changes. *Nat Biotechnol*. 2004;22:1383-1391.

6. Laue T. Biophysical studies by ultracentrifugation. *Curr Opin Struct Biol*. 2001;11:579-583.

7. Lebowitz J, Lewis MS, Schuck P. Modern analytical ultracentrifugation in protein science: a tutorial review. *Protein Sci*. 2002;11:2067-2079.

8. Dam J, Schuck P. Calculating sedimentation coefficient distributions by direct modeling of sedimentation velocity concentration profiles. *Methods Enzymol*. 2004;384:185-212.

9. Laue T. Analytical ultracentrifugation: a powerful 'new' technology in drug discovery. *Drug Discov Today: Technol*. 2004;1:309-315.

10. Philo JS. Analytical ultracentrifugation. In: Jiskoot W, Crommelin D, eds. *Methods for Structural Analysis of Protein Pharmaceuticals*. Arlington, VA: AAPS Press; 2005:379-412.

11. Svedberg T, Pederson KO. *The Ultracentrifuge*. London, UK: Oxford University Press; 1940.

12. Lewis MS, Weiss GH, eds. *Fifty Years of the Ultracentrifuge*. Amsterdam, The Netherlands: North-Holland Publishing Company; 1976.

13. Schachman HK. Is there a future for the ultracentrifuge? In: Harding SE, Rowe AJ, Horton JC, eds. *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. Cambridge, UK: Royal Society for Chemistry, Redwood Press; 1992:3-15.

14. Bowen JT. *An Introduction to Ultracentrifugation*. London, UK: John Wiley & Sons; 1970:1-14.

15. Van Holde KE, Hansen JC. Analytical ultracentrifugation from 1924 to the present: a remarkable history. *Chemtracts: Biochem Mol Biol*. 1998;11:933-943.

16. Ranby B. *Physical Chemistry of Colloids and Macromolecules*. Oxford, UK: Blackwell Scientific; 1987.

17. Righetti PG. Bioanalysis: its past, present, and some future. *Electrophoresis*. 2004;25:2111-2127.

18. Porath J, Flodin P. Gel filtration: a method for desalting and group separation. *Nature*. 1959;183:1657-1659.

19. Porath J. Some recently developed fractionation procedures and their application to peptide and protein hormones. *Pure Appl Chem*. 1963;6:233-240.

20. Ornstein L. Disc electrophoresis, I: background and theory. *Ann N Y Acad Sci*. 1964;121:321-349.

21. Davis BJ. Disc electrophoresis, II: method and application to human serum proteins. *Ann N Y Acad Sci*. 1964;121:404-427.

22. Laemmli UK. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature*. 1970;227:680-685.

23. Karas M, Bachmann D, Bahr U, Hillenkamp F. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int J Mass Spectrom*. 1987;78:53-68.

24. Fenn JB, Mann M, Meng CK, Wong SF, Whithouse CM. Electrospray ionization for mass spectrometry of large biomolecules. *Science*. 1989;246:64-71.

25. Schachman HK. Analytical ultracentrifugation reborn. *Nature*. 1989;341:259-260.

26. Giebeler R. The Optima XL-A: a new analytical ultracentrifuge with a novel precision absorption optical system. In: Harding SE, Rowe AJ, Horton JC, eds. *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. Cambridge, UK: Royal Society for Chemistry, Redwood Press; 1992:16-25.

27. Ralston G. The renaissance of the analytical ultracentrifuge. *Today's Life Science*. 1991;3:43-50.

28. Harding SE. Analytical ultracentrifugation and the genetic engineering of macromolecules. *Biotechnol Genet Eng Rev*. 1993;11:317-356.

29. Harding SE. The analytical ultracentrifuge spins again. *Trends Analyt Chem*. 1994;13:439-446.

30. Shire SJ. Analytical ultracentrifugation and its use in biotechnology. In: Schuster TM, Laue TM, eds. *Modern Analytical Ultracentrifugation: Acquisition and Interpretation of Data for Biological and Synthetic Polymer Systems*. Boston, MA: Birkhauser 1994:261-267.

31. Schuster TM, Toedt JM. New revolution in the evolution of analytical ultracentrifugation. *Curr Opin Struct Biol*. 1996;6:650-658.

32. Stafford WF, III. Sedimentation velocity spins a new weave for an old fabric. *Curr Opin Biotechnol*. 1997;8:14-24.

33. Liu J, Shire SJ. Analytical ultracentrifugation in the pharmaceutical industry. *J Pharm Sci*. 1999;88:1237-1241.

34. Varley PG, Brown AJ, Dawkes HC, Burns NR. A case study and use of sedimentation equilibrium analytical ultracentrifugation as a tool for biopharmaceutical development. *Eur Biophys J*. 1997;25:437-443.

35. Stafford WF, III. Boundary analysis in sedimentation transport experiments: a procedure for obtaining sedimentation coefficient distributions using the time derivative of the concentration profile. *Anal Biochem*. 1992;203:295-301.

36. Philo JS. Measuring sedimentation, diffusion and molecular weight of small molecules by direct fitting of sedimentation velocity concentration profiles. In: Schuster TM, Laue TM, eds. *Modern Analytical Ultracentrifugation: Acquisition and Interpretation of Data for Biological and Synthetic Polymer Systems*. Boston, MA: Birkhauser; 1994:156-170.

37. Cole JJ, Hansen JC. Analytical ultracentrifugation as a contemporary biomolecular research tool. *J Biomol Tech*. 1999;10:163-176.

38. Demeler B, Saber H, Hansen JC. Identification and interpretation of complexity in sedimentation velocity boundaries. *Biophys J*. 1997;72:397-407.

39. Behlke J, Ristau O. Molecular mass determination by sedimentation velocity experiments and direct fitting of the concentration profiles. *Biophys J*. 1997;72:428-434.

40. Demeler B, Saber H. Determination of molecular parameters by fitting sedimentation data to finite-element solutions of the Lamm equation. *Biophys J*. 1998;74:444-454.

41. Reversible associations in structural and molecular biology. RASMB Web site. Available at: http://www.bbri.org/RASMB/rasmb.html. Accessed January 15, 2006.

42. Harding SE, Rowe AJ, Horton JC, eds. *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. Cambridge, UK: Royal Society for Chemistry, Redwood Press; 1992.

43. Schuster TM, Laue TM, eds. *Modern Analytical Ultracentrifugation: Acquisition and Interpretation of Data for Biological and Synthetic Polymer Systems*. Boston, MA: Birkhauser;1994.

44. Hansen JC, Lebowitz J, Demler B. Analytical ultracentrifugation of complex macromolecular systems. *Biochemistry*. 1994;33:13155-13163.

45. Hensley P. Defining the structure and stability of macromolecular assemblies in solution: the reemergence of analytical ultracentrifugation as a practical tool. *Structure*. 1996;4:367-373.

46. Laue TM, III, Stafford WF, III. Modern applications of analytical ultracentrifugation. *Annu Rev Biophys Biomol Struct*. 1999;28:75-100.

47. Correia JJ. Sedimentation velocity analysis methods: what, when, why? *Chemtracts: Biochem Mol Biol*. 1998;13:944-949.

48. Stafford WF, III. Analysis of reversibly interacting macromolecular systems by time derivative sedimentation velocity. *Methods Enzymol*. 2000;323:302-325.

49. Carruthers LM, Schirf VR, Demeler B, Hansen JC. Sedimentation velocity analysis of macromolecular assemblies. *Methods Enzymol*. 2000;321:66-80.

50. Demeler B, Behlke J, Ristau O. Molecular parameters from sedimentation velocity experiments: whole boundary fitting using approximate and numerical solutions of Lamm equation. *Methods Enzymol*. 2000;321:38-66.

51. Schuck P. Sedimentation analysis of non-interacting and self-associating solutions using numerical solutions to the Lamm equation. *Biophys J*. 1998;75:1503-1512.

52. Schuck P, Demeler B. Direct sedimentation analysis of interference optical data in analytical ultracentrifugation. *Biophys J*. 1999;76:2288-2296.

53. Schuck P. Sedimentation equilibrium: an analysis of interference optical data by systematic noise decomposition. *Anal Biochem*. 1999;272:199-208.

54. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophys J*. 2000;78:1606-1619.

55. Schuck P. Rossmanith P. Determination of the sedimentation coefficient distribution by least-squares boundary modeling. *Biopolymers*. 2000;54:328-341.

56. Solovyova A, Schuck P, Costenaro L, Ebel C. Non-ideality by sedimentation velocity of halophilic malate dehydrogenase in complex solvents. *Biophys J*. 2001;81:1868-1880.

57. Schuck P, Perugini MA, Gonzales NR, Howlett GJ, Schubert D. Size-distribution analysis of proteins by analytical ultracentrifugation: strategies and application to model systems. *Biophys J*. 2002;82:1096-1111.

58. Schuck P. On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation. *Anal Biochem*. 2003;320:104-124.

59. Vistica J, Dam J, Balbo A, et al. Sedimentation equilibrium analysis of protein interactions with global implicit mass conversation constraints and systematic noise decomposition. *Anal Biochem*. 2004;326:234-256.

60. Schuck P. A model for sedimentation in inhomogeneous media, I: dynamic density gradient from sedimenting co-solutes. *Biophys Chem*. 2004;108:187-200.

61. Schuck P. A model for sedimentation in inhomogeneous media, II: compressibility of aqueous and organic solvents. *Biophys Chem*. 2004;108:201-214.

62. Dam J, Velikovsky CA, Mariuzza RA, Urbanke C, Schuck P. Sedimentation velocity analysis of heterogeneous protein-protein interactions: Lamm equation modeling and sedimentation coefficient distributions c(s). *Biophys J*. 2005;89:619-634.

63. Dam J, Schuck P. Sedimentation velocity analysis of heterogeneous protein-protein interactions: sedimentation coefficient distributions c(s) and asymptotic boundary profiles for Gilbert-Jenkins theory. *Biophys J*. 2005;89:651-666.

64. Scott DJ, Harding SE, Rowe AJ, eds. *Modern Analytical Ultracentrifugation: Techniques and Methods*. Cambridge, UK: Royal Society of Chemistry; 2005.

65. Brown PH, Schuck P. Macromolecular size-and-shape distributions by sedimentation velocity analytical ultracentrifugation. *Biophys J*. 2006;90:4651-4661.

66. Philo JS. Improved methods for fitting sedimentation coefficient distributions derived by time-derivative techniques. *Anal Biochem*. 2006;354:238-246.

67. Cleland JL, Powell MF, Shire SJ. The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. *Crit Rev Ther Drug Carrier Syst*. 1993;10:307-377.

68. Costantino HR, Langer R, Klibanov AM. Solid-phase aggregation of proteins under pharmaceutically relevant conditions. *J Pharm Sci*. 1994;83:1662-1669.

69. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solutions: mechanism and driving forces in nonnative protein aggregation. *Pharm Res*. 2003;20:1325-1336.

70. Wang W. Protein aggregation and its inhibition in biopharmaceuticals. *Int J Pharm*. 2005;289:1-30.

71. Lakowicz JR, Weber G. Quenching of protein fluorescence by oxygen: detection of structural fluctuations in macromolecules. *Biochemistry*. 1973;12:4161-4170.

72. Volkin DB, Mack H, Middaugh CR. Degradative covalent reactions important to protein stability. In: Shirley BA, ed. *Methods in Molecular Biology: Protein Stability and Folding, Theory and Practice.* vol. 40. Totowa, NJ: Humana Press; 1995:35-63.

73. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int J Pharm*. 1999;185:129-188.

74. Patten PA, Schellekens H. The immunogenicity of biopharmaceuticals: lessons learned and consequences for protein drug development. In: Brown F, Mire-Sluis AR. eds. *Immunogenicity of Therapeutic Biological Products.* vol. 112. Basel, Switzerland: Karger; 2003:81-97.

75. Rosenberg AS, Worobec AS. A risk-based approach to immunogenicity concerns of therapeutics protein products, Part 2: considering host-specific and product-specific factors impacting immunogenicity. *Biopharm Int*. 2004;17:34-42.

76. Bucciantini M, Glannoni E, Chiti F, et al. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature*. 2002;416:507-511.

77. Shire SJ, Shahrokh Z, Liu J. Challenges in the development of high protein concentration formulations. *J Pharm Sci*. 2004;93:1390-1402.

78. Moore JMR, Patapoff TW, Cromwell EM. Kinetics and thermodynamics of dimer formation and dissociation for a recombinant humanized monoclonal antibody to vascular endothelial growth. *Biochemistry*. 1999;38:13960-13967.

79. Minton AP. The effect of volume occupancy upon the thermodynamic activity of proteins: some biochemical consequences. *Mol Cell Biochem*. 1983;55:119-140.

80. Zimmerman SB, Minton AP. Macromolecular crowding: biochemical, biophysical, and physiological consequences. *Annu Rev Biophys Biomol Struct*. 1993;22:27-63.

81. Minton AP. Molecular crowding: analysis of the effects of high concentrations of inert cosolutes on the biochemical equilibria and rate in terms of volume exclusion. *Methods Enzymol*. 1998;295:127-149.

82. van den Berg B, Ellis RJ, Dobson CM. Effects of macromolecular crowding on protein folding and aggregation. *EMBO J*. 1999;18:6927-6933.

83. Gamble CN. The role of soluble aggregates in the primary immune response of mice to human gamma globulin. *Int Arch Allergy Appl Immunol*. 1966;30:446-455.

84. Bachmann M, Rohrer U, Kundig T, Burki K, Hengarthner H, Zinkernagel RM. The influence of antigen organization on B cell responsiveness. *Science*. 1993;262:1448-1451.

85. Braun A, Kwee L, Labow M, Alsenz J. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. *Pharm Res*. 1997;14:1472-1478.

86. Moore W, Leppert P. Role of aggregated human growth hormone (hGH) in development of antibodies to hGH. *J Clin Endocrinol Metab*. 1980;51:691-697.

87. Laue TM. Sedimentation equilibrium as a thermodynamic tool. *Methods Enzymol*. 1995;259:427-452.

88. Kar SR, Kingsbury JS, Lewis MS, Laue TM, Schuck P. Analysis of transport experiments using pseudo-absorbance data. *Anal Biochem*. 2000;285:135-142.

89. Cantor CR, Schimmel PR. *Biophysical Chemistry, Part II: Techniques for the Study of Biological Structure and Function.* San Francisco, CA: W.H. Freeman & Co; 1980:595.

90. Lamm O. Die differentialgleichung der ultazentrifugierung. *Ark Mat Astr Fys Part B*. 1929;21B:1-4.

91. Cantor CR, Schimmel PR. *Biophysical Chemistry, Part II Techniques for the study of biological structure and function*. San Francisco, CA: W.H. Freeman & Company;1980:601-602.

92. Schachman HK. *Ultracentrifugation in Biochemistry.* New York, NY: Academic Press;1959.

93. Goldberg RJ. Sedimentation in the ultracentrifuge. *J Phys Chem*. 1953;57:194-202.

94. Balbo A, Schuck P. Analytical ultracentrifugation in the study of protein self-association and heterogeneous protein-protein interactions. Protein-Protein Interactions. Office of Research Services Web Site. Available at: http://dbeps.ors.od.nih.gov/pbr_AUC.htm. Accessed February 2, 2006.

95. Balbo A, Schuck P. Analytical ultracentrifugation in the study of protein self-association and heterogeneous protein-protein interactions: protocol for velocity and equilibrium sedimentation.Office of Research Services Web site. Available at: http://dbeps.ors.od.nih.gov/pbr_AUC.htm. Accessed February 2, 2006.

96. Maizel JV, White DO, Schaff MD. The polypeptides of adenovirus, II: soluble protein, cores, top components and structure of the virion. *Virology*. 1968;36:126-136.

97. Vellekamp G, Porter FW, Sutjipto S, et al. Empty capsids in column-purified recombinant adenovirus preparations. *Hum Gene Ther*. 2001;12:1923-1936.

98. Schachman HK. Sedimentation Velocity. In: *Ultracentrifugation in Biochemistry.* New York, NY: Academic Press; 1959:151-157.

99. Schachman HK. Experimental Aspects. In:*Ultracentrifugation in Biochemistry.* New York, NY: Academic Press; 1959:41-47.

100. Laue TM. Choosing which optical system of the optima XL-I analytical ultracentrifuge to use. Available at: http://beckmancoulter.com/literature/bioresearch/1821a(a).pdf. Accessed February 1, 2006.

101. Machtle W. High-resolution, submicron particle size distribution analysis using gravitational-sweep sedimentation. *Biophys J*. 1999;76:1080-1091.

102. Stafford WF, III, Braswell EH. Sedimentation velocity, multi-speed method for analyzing polydisperse solutions. *Biophys Chem*. 2004;108:273-279.

103. Philo JS. Characterizing the aggregation and conformation of protein therapeutics. Available at: http://beckmancoulter.com/literature/bioresearch/AR-9663A.pdf. Accessed February 3, 2006.